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Detection of gsp oncogene in growth hormone-secreting pituitary adenomas and the study of clinical characteristics of acromegalic patients with gsp-positive pituitary tumors

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Objective To investigate the incidence and clinical characteristics of gsp oncogene positive growth hormone-secreting adenomas of Chinese acromegalic patients.

Methods Continuously 40 patients were studied. Serum hormone levels of pituitary and target glands were measured and growth hormone (GH)-TRH stimulating tests were done before transsphenoidal or transfrontal hypophysectomy. Deoxyribonucleic acid (DNA) was extracted from the frozen tumor tissue, and the DNA fragment encompassing codon 201 and 227 of the Gsα gene was amplified by polymerase chain reaction (PCR). Point mutations at codon 201 and 227 were detected using PCR direct sequencing method in order to get the incidence of gsp oncogene in GH secreting adenomas.

Results Of 40 tumors studied, 22 (55%) were gsp positive. The point mutation from CGT (Arg) to TGT (Cys) at codon 201 was detected in 21 pituitary tumors, but the point mutation from CAG (Gln) to CTG (Leu) at codon 227 of the Gsα gene was found in only 1 tumor. All of the point mutations are heterozygous. The number of gsp positive patients which have 30% or more decrease of serum GH concentration after glucose inhibition is less than that of gsp negative patients ($P = 0.042$). Compared to gsp negative patients, most of gsp positive patients showed paradoxical response to TRH stimulation ($P = 0.002$). There were more gsp positive patients with the tumor diameter less than 25 mm ($P = 0.029$) and with normal GH levels in OGTT after surgery ($P = 0.007$).

Conclusions Gsp mutation is one of the major intrinsic defects in the pathogenesis of growth hormone-secreting pituitary tumors and the identification of gsp mutation can be a reference for classification and prognosis of GH tumors.

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Growth hormone (GH)-secreting pituitary adenoma is one of the most common hormone-secreting tumors. The etiology of GH adenoma is still not fully known. In recent years, several research groups have reported the point mutation at codon 201 and 227 of stimulating GTP binding protein α-subunit (Gsα).[1-8] The mutant form of Gsα is constitutively activated and continuously stimulates the adenylyl cyclase (AC) signaling pathway, which results in persistent increased GH secretion and hyperplasia of GH tumor cells.[1,2] The mutant form of Gsα gene is also called gsp oncogene.[2] The gsp oncogene has been identified in about 35% – 43% of GH-secreting adenomas in western countries[4-7] and Korea,[8] but a considerably lower incidence of gsp oncogene has been reported in Japanese patients.[9,10] Furthermore, the clinical characteristics of the tumors expressing gsp oncogene are contradictory in the previous studies. The purpose of this study was to investigate the incidence of gsp oncogene in GH tumors of Chinese acromegalic patients and to elucidate the clinical characteristics of Chinese patients with gsp-positive GH tumors.

METHODS

Identification of gsp mutations

DNA was extracted from 40 cases of GH-secreting pituitary tumors and peripheral blood leukocytes of 3 acromegalic patients using phenol-chloroform extraction method.[11] A single-stranded DNA fragment encompassing codons 201 and 227 of the Gsα gene was generated from each of the DNA preparations by polymerase chain reaction (PCR). In PCR, genomic DNA (200-500 ng) was mixed with 5' and 3' primers (0.5 μmol/L each; the 5'-primer sequence is 5'-GTGTGCAAAACCCCTCCCCACCAG-3'; the 3'-primer sequence is 5'-CCAAGAGCGTGAGCAGCGACCCT-3'),

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$MgCl_2$ 1.5 mmol/L, deoxynucleotide triphosphates (100 μmol/L each), Tris-HCl 10 mmol/L, KCl 50 mmol/L and 2.0 U of Taq DNA polymerase (Promega, U.S.A.) in a total volume of 50 μl. The creation of the sequences of two primers referred to the Gsα sequence was reported previously.[12] The template DNA was initially denatured at 95℃ for 3 minutes, followed by 30 successive cycles at 94℃ (20 seconds) and 70℃ (40 seconds). Before the reaction completed, 10-minutes extention was performed. The amplified fragment was purified by argarose gel electrophoresis, and the resected gel containing the fragment was passed through a minicolumn (product of Promega Company) to remove the excessive primers and dNTPs. The sequencing reaction was performed on the purified PCR product by using a di-deoxy sequencing kit (Promega) with 32^P-labled deoxynucleotide adenosine triphosphate as a label. Sequencing reaction products were loaded onto an 7% polyacrylamide sequencing gel. After electrophoresis, the gel was transferred to filter paper, dried and autoradiographed. The base change in mutation of each case was confirmed by a second PCR amplification and sequencing.

Clinical evaluation

Continuous 40 acromegalic patients were selected. Among them, 20 were males and 20 females. The criteria for selection were the same as that of documented diagnosis of GH-secreting pituitary adenoma. All of the tumors were removed by transsphenoidal or transfrontal hypophysectomy. The pathological diagnosis of all the removed tumors is pituitary adenoma, and 35 out of the 40 tumors were proven by immune-histochemical staining to be GH-secreting adenomas. The clinical presentations of the patients were carefully evaluated preoperatively. In TRH-stimulating test, 200 μg TRH was administered intravenously. Blood sampling was taken at 0, 20, 30, 60, 90 minutes. The criterion for a positive response to TRH is that the serum GH levels after TRH injection increased more than 50% above the baseline value.[13] The size of pituitary tumor was measured by MRI or CT image or estimated by surgeon during the operation.

Statistics

Quantitative data were expressed as $\bar{x} \pm s$, analysis of quantitative data was performed using Student's t test, and qualitative data were analyzed by the Chi-square test.

RESULTS

gsp mutation detection of GH secreting adenoma

Of the 40 tumors studied, 22 (55%) were gsp-positive. The mutation was located at codon 201 in 21 cases, in which a CGT to TGT transition was found, resulting in a mutation from arginine to cysteine. In the other one gsp-positive tumor, the mutation altered glutamine to leucine at codon 227 by a CAG to CTG single base transition. All of these mutations were apparently heterozygous, i.e., one allele displayed the base change and the other displayed the wild-type sequence. This resulted in a "double-base" finding on the sequencing gels (Figs. 1 and 2). The genomic DNA prepared from peripheral leukocytes of 2 gsp-positive and 1 gsp-negative patients showed only wild-type sequence.

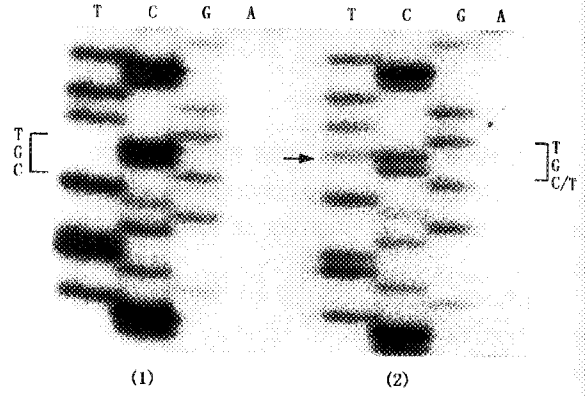

Fig. 1. Nucleotide sequences of codon 201 of Gsα gene. The sequences were determined by the polymerase chain reaction-direct sequencing method using frozen pituitary tumor tissue. (1) The normal sequence of codon 201 is CGT. (2) The mutant sequence of codon 201: a T band (arrowed) exists at the same level of C band; there are two sequences in codon 201, the normal CGT and the mutant TGT.

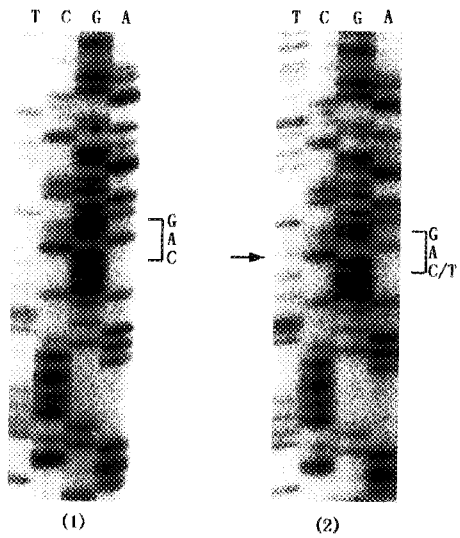

Fig. 2. Nucleotide sequences of codon 227 of Gsα gene. (1) The normal sequence of codon 227 is CAG. (2) The mutant sequence of codon 227: a very weak T band (arrowed) exists at the same level of A band; there are two sequences in codon 227, the normal CAG and the mutant CTG.

Clinical characteristics of gsp-positive acromegalic patients

The comparison of the clinical characteristics between the gsp-positive and gsp-negative acromegalic patients is shown on Table. There was no significant difference in

Table. The comparison of clinical characteristics between gsp-positive and gsp-negative acromegalic patients

Group	n	Sex (M/F)	Age (yrs)	Duration (yrs)	Basal GH (μg/L)	PRL (μg/L)	α-subunit (μg/L)	Tumor diameter (mm)
gsp-positive	22	11/11	41.9 ± 10.9	7.2 ± 5.4	58.0 ± 53.9	21.9 ± 34.0	11.8 ± 24.3	22.6 ± 8.7
(n)			(22)	(22)	(20)	(22)	(18)	(22)
gsp-negative	18	9/9	36.1 ± 11.3	5.6 ± 2.9	92.7 ± 82.9	19.2 ± 25.3	0.6 ± 6.8	26.3 ± 10.4
(n)			(18)	(18)	(15)	(18)	(12)	(18)

average age, male to female ratio and disease duration between the two groups. The frequency of symptoms did not differ obviously either. The average serum levels of basal GH of gsp-positive patients did not differ significantly from that of gsp-negative group ($P = 0.158$). But in preoperative OGTT, the number of gsp-positive patients with more than 30% decline of GH concentration after glucose loading was more than that of gsp-negative patients ($P = 0.042$). The number of patients with positive TRH-stimulating test was also significantly more than negative groups ($P = 0.002$). There were no obvious differences in serum PRL and α-subunit levels between the gsp-positive and gsp-negative patients.

No significant difference in average tumor diameter of gsp-positive and gsp-negative patients was found, but more gsp-positive patients had tumor diameter less than 25 mm ($P = 0.029$). As to the degree of tumor invasiveness, there was no significant difference between the gsp-positive and gsp-negative group. In postoperative OGTT, the serum GH levels of more gsp-positive patients were in the normal range ($P = 0.007$).

The only one patient with a point mutation at codon 227 is a 33-year-old female, the tumor is a microadenoma. She had negative response to TRH stimulation and had normal GH level at 2 years after the operation.

DISCUSSION

Stimulating GTP binding protein (Gs) takes part in a cascade of cellular events mediating cell hyerplasia and hormone secretion in endocrine tissues. In the process, Gsα activates adenylyl cyclase (AC) by combining with GTP, and becomes inactivated by hydrolyzing GTP to GDP. Mutations at either codon 201 and 227 of Gsα were proven to produce protein with very low GTPase activity in the transfected cells,[2] and thus result in stabilization of the mutant Gsα in their active GTP-bound state. The constitutively active Gsα activates adenylyl cyclase (AC) persistently to produce high levels of intracellular cAMP, which in turn results in continuous GH hypersecretion and enhanced somatotroph proliferation. It was reported that eight of 8 gsp-positive GH tumors contain high level of intracellular cAMP,[3] which proved that constitutive activation of the adenylyl cyclase signalling pathway in GH-secreting tumors and the presence of gsp mutation correlated with one another.

Our previous study has shown that 81% of GH adenoma cells in vitro culturing have abnormal GHRH receptor and/or Gs protein.[14] The present study further found that 55% of GH tumors contain gsp oncogene, which informed us that the gsp mutation plays an important role in a subset of human GH-secreting tumors. The frequency of the Gsα mutation has been reported to be 34.6% – 42.0% in western studies[3-7] and 42.9% in a Korean study,[8] but only 4.4% – 9.3% in Japanese studies.[9,10] The incidence of 55% in the present study is near that in the report of western countries and Korea. This study suggests that the low frequency of gsp oncogene in Japanese acromegalic patients is not likely explained by the racial differences. Further studies are needed to investigate the reason for the difference between the gsp frequency of Japanese studies and that of other studies. No gsp oncogene was detected in the peripheral leukocytes of two patients with gsp-positive pituitary tumor. Some studies[3,8] also showed that gsp mutations took place only in tumor cells and not in peripheral leukocytes. This indicates that gsp mutations are an important intrinsic defect of pituitary tumor cell. So the higher surgical cure rate of gsp-positive tumor can be explained by the removal of the causal gsp oncogene.

Several authors[6-8] have investigated the correlations between mutations of the Gsα gene and the clinical features of GH adenomas. In our study, gsp-positive GH tumors secret GH more autonomously. The serum GH levels of gsp-positive patients were not inhibited by glucose as much as that of gsp-negative ones, which is contraindicated with the results of Landis et al[7] and Yang et al.[8] However, our result seems to be more easily explained. The mutant Gsα acts by mimicking continuous stimulation of pituitary somatotrophs by GHRH, and it is not so sensitive to the normal regulatory mechanism. It has been proven that there are contact and functioning TRH receptors on some of the GH tumor cells.[15] This can explain the phenomenon that most of gsp-positive tumors respond well to TRH stimulation.

Clinical and pathological studies distingush two major subgroups of patients with GH-secreting pituitary tumors.[16] The first subgroup is characterized by small, slowly growing, well differentiated, granulated tumors, and an indolent clinical course; some authors reported the better response of GH to somatostatin in this subgroup.[17] The second subgroup is characterized by large, rapidly growing, poorly differentiated, and sparsely granulated tumors, recurring more

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frequently and with higher basal serum GH level.[16] The clinical characteristics of gsp-positive GH tumor in the present study suggested that these tumors belong to the first subgroup. And in this study, more higher surgical cure rate was found in gsp-positive GH tumors. So the detection of gsp oncogene may aid in classification and may be a useful reference for the prognosis of GH tumors.

We analyzed Gsα gene mutations in the GH adenomas of 40 patients with acromegaly using PCR-direct sequencing method rather than dot-blot hybridization with a specific oligonucleotide probe or single-strand conformational polymorphism (SSCP) analysis, because the latter two methods have shortcomings with regards to sensitivity and specificity.[18] Owing to the hyterozygosity of the mutant gene, the sequence bands obtained by PCR direct sequencing method are more easily to be read than that of autonomous sequencing.

We concluded that Gsα gene mutation of GH secreting adenomas in Chinese acromegalic patients are as common as that of Korean and Caucasian patients. The gsp-positive tumors are smaller in tumor size, more autonomous in GH secretion and with higher surgical cure rate. The detection of gsp oncogene may be useful for tumor classification and may serve as a useful prognostic factor.

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